CTLA-4 Engagement Inhibits IL-2 Accumulation and Cell Cycle Progression upon Activation of Resting T Cells

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Summary

While interactions between CD28 and members of the B7 family costimulate and enhance T cell responses, recent evidence indicates that the CD28 homologue CTLA-4 plays a downregulatory role. The mechanism by which this occurs is not clear, but it has been suggested that CTLA-4 terminates ongoing responses of activated T cells, perhaps by induction of apoptosis. Here we demonstrate that CTLA-4 engagement by antibody cross-linking or binding to B7 inhibits proliferation and accumulation of the primary T cell growth factor, IL-2, by cells stimulated with anti-CD3 and anti-CD28. This inhibition is not a result of enhanced cell death. Rather it appears to result from restriction of transition from the G1 to the S phase of the cell cycle. Our observation that upregulation of both the IL-2R alpha chain and the CD69 activation antigen are inhibited by CTLA-4 engagement supplies further evidence that CTLA-4 restricts the progression of T cells to an activated state. Together this data demonstrates that CTLA-4 can regulate T cell activation in the absence of induction of apoptotic cell death.

It has by now become generally accepted that the outcome of T cell antigen receptor occupancy is strongly influenced by costimulatory signals provided by interactions between CD28 on the T cell surface and its ligands B7-1 (CD80) and B7-2 (CD86) on the antigen-presenting cell (1–3). These costimulatory signals enhance production of several lymphokines, especially IL-2, and greatly increase proliferation. An additional effect of CD28 costimulation is the induction of expression of bcl-xL, which has been associated with enhanced T cell survival (4). Recent findings suggest that the CD28 homologue CTLA-4 which, like CD28, binds members of the B7 family, also plays a very important role in the outcome of T cell activation.

A role for CTLA-4 in T cell activation was originally suggested by the fact that antibodies to CTLA-4, while not themselves costimulatory, augmented proliferation of T cells costimulated by anti-CD28 (5). It was subsequently shown that both intact anti-CTLA-4 and its Fab fragments enhanced T cell proliferative responses in vitro, and that both soluble anti-CTLA-4 and anti-B7 antibodies enhanced proliferation of T cells activated by anti-CD3 and costimulated with anti-CD28 (6, 7). On the other hand, anti-CTLA-4 antibodies inhibited T cell proliferation when Fc receptor-mediated cross-linking was available or when cross-linked or presented in immobilized form together with anti-CD3 and anti-CD28 (6, 7). Together, these results suggested an inhibitory role for CTLA-4. This notion was further supported by demonstrations that in vivo blockade of CTLA-4/B7 interaction by either intact CTLA-4 antibody or its Fab fragment increased the expansion of T cells reactive with a specific peptide antigen or the superantigen Staphylococcus enterotoxin B (SEB) (8, 9). Finally, recent reports have documented a rampant T cell lymphocytic proliferative disorder with early lethality in CTLA-4-deficient mice (10, 11). Together, these findings demonstrate that CD28 and CTLA-4 have opposing effects on T cell activation, and that CTLA-4 has a very important role in downregulation of T cell responses.

The mechanism of CTLA-4 inhibition of T cell responses is not clear. Since CTLA-4 expression reaches maximal levels by T cells 2–3 d after activation, it has been suggested that its role is to terminate T cell responses at that point and facilitate the generation of memory T cells ready to respond to antigen after decay of CTLA-4 expression (1, 3, 12, 13). Alternatively it has been suggested that CTLA-4 signaling might terminate T cell responses by inducing cell death by an apoptotic mechanism (14). In support of this possibility, it has been reported that an anti-CTLA-4 antibody, although not the known B7 ligands, can induce cell death in activated T cell clones (15).

Here we report a kinetic analysis of the effects of CTLA-4 ligation on proliferation, IL-2 production, cell death, cell cycle progression, and the appearance of T cell activation markers. By blockade of CTLA-4/B7 interactions and by antibody-mediated cross-linking we demonstrate that CTLA-4 ligation inhibits T cell proliferation and IL-2 accumulation. These responses are not precipitously terminated once established, but are delayed and diminished. There is no evi-

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/06/2533/09 $2.00
Volume 183 June 1996 2533–2540

dence for induction of cell death by CTLA-4 cross-linking at any time during the response. Inhibition of the proliferative response appears to be a consequence of arrest in the G0/G1 stage of the cell cycle perhaps as a consequence of diminished IL-2 production and inhibition of expression of the IL-2 receptor alpha chain. Finally, we show that CTLA-4 ligation also inhibits expression of CD69, an early marker of T cell activation. These data are not consistent with inhibition by induction of cell death. Rather, they suggest that CTLA-4 can restrict the transition of T cells to an activated state.

Materials and Methods

Antibodies and Reagents. Antibodies used for activation were: anti-CD3 (hybridoma 500A2 [16]), anti-CD28 (hybridoma 37.N.51.1 [17]), anti-CTLA-4 (hybridoma 9H10.11G3 [7]), and anti-Vγ3 (hybridoma 536 [18]). CTLA-4Ig was a kind gift of Drs. K. Karljialeinen and P. Lane (19). APC and CD8 depletion was achieved using anti-Class II MHC (hybridomas 28-16-8s [20] and BP107 [21]), and anti-CD8 antibodies (hybridoma 3.155 [22]). Sulfate polystyrene latex microspheres of 5 ± 0.1 μM mean diameter were obtained from Interfacial Dynamics Corp. (Portland, OR).

Preparation of CD4+ T Lymphocytes. Lymph node cells were isolated from 6–8-wk-old BALB/c mice obtained from National Cancer Institute (Bethesda, MD). Isolated lymphocytes were obtained by the mincing of tissue and filtration of the resulting suspension through nytex. Enriched CD4+ T cell preparations were obtained by treatment with complement, anti–Class II antibodies, and anti-CD8 antibodies as described (23). Typical preparations were 95% CD4+ with less than 0.75% B220 positive cells.

Activation of CD4+ T Cells Using Immobilized Anti-CD3. Round-bottom 96-well plates were coated with anti-CD3 at 0.1 μg/ml in 50-μl vol for 2 h at 37°C, then washed extensively and blocked for 30 min at 37°C with complete RPMI-1640 (containing 10% FCS, 50 μM β-mercaptoethanol, 2 mM glutamine, and 50 μg/ml gentamycin). T cells were added at 1×10^5 per well in 200 μl of complete RPMI-1640 and all cultures were incubated at 37°C in 5% CO_2. Where indicated anti-CD28 was added at 10 μg/ml, CTLA-4Ig was added at 5 μg/ml, and control or anti-CTLA-4 FAb fragments were added at 50 μg/ml. 12 h before harvest, wells were pulsed with 20 μl of complete RPMI containing 1 μCi of [^{3}H]thymidine. Plates were harvested to glass filter mats and ^{3}H incorporation was measured using a gas-phase counter (Packard, Meriden, CT).

Activation of T cells Using Latex Microspheres. Latex microspheres (beads) were coated as described (7). Briefly, 1×10^7 beads/ml were suspended in PBS with the indicated antibodies and incubated for 1.5 h at 37°C, followed by washing with PBS and blocking with 10% FCS. Anti-CD3 was added at 0.5 μg/ml, anti-CD28 was added at 1 μg/ml, anti-CTLA-4 was added at 4 μg/ml, and binding solutions were normalized with control antibody 536 to maintain a constant total antibody concentration of 6 μg/ml during binding. T cells (1×10^5/200 μl) were cultured with 1×10^5 beads in a total volume of 200 μl/well. Round-bottom 96-well plates were used for all assays. Cultures were incubated at 37°C in 5% CO_2 and pulsed with 1 μCi of [^{3}H]thymidine for the final 12 h before harvesting. The inhibitory action of CTLA-4 appears specific to anti-CTLA-4 antibodies as other T cell binding antibodies including anti-L selectin (Mel-14), anti-Thy1.2 and irrelevant antibodies show either no effect or augmentatory effects when coimmobilized with anti-CD3 and anti-CD28.

Analysis of Cell Viability. T cells were cultured identically as for proliferation assays. At the indicated times, cell viability was assessed by the addition of one-tenth volume of 0.4% trypan blue (Sigma, St. Louis, MO) and cell numbers determined using a hemocytometer. 10^{-4} ml of each culture was counted from duplicate wells and the value for this volume was multiplied by 2 to obtain a value for the percent of input (50×10^4 cells/ml was input). Standard deviations were always less than 10%.

Cell Cycle Analysis. Propidium iodide analysis of cell cycle status was performed as previously described (24). Briefly, cells were activated as described using microspheres in 96-well plates as described for proliferation assays. At the indicated times, three identical wells (3×10^5 input at the beginning of culture per sample) were harvested, washed in PBS, and fixed with 1.0 ml of 80% ethanol. Cells were incubated on ice for 30 min, pelleted by centrifugation and resuspended in 0.4 ml of an aqueous solution containing 0.1% Triton X-100, 0.1 mM EDTA, 0.05 mg/ml RNaseA (50 U/mg), and 50 μg/ml propidium iodide. Samples were stored on ice in the dark until analysis and each sample was analyzed at a constant flow rate for 2 min. Data was analyzed using a Coulter EPICS system (Coulter Corp., Hialeah, FL).

IL-2 Determination. An ELISA was used to detect IL-2 in cell supernatants. Briefly, capture antibodies were coated at 1 μg/ml onto Corning (Corning, NY) ELISA plates in Borate Buffer (0.2 M Na Borate, pH 8.0) for 2 h at 37°C. These plates were then washed extensively, blocked with 0.4% Gelatin/PBS for 30 minutes, and then T cell culture supernatants (50 μl) were added and incubated for 2 h at 37°C. Plates were again washed and biotinylated detection antibodies were added in PBS/0.5% Tween and incubated for 1 h at 37°C. Plates were again washed and 50 μl of a solution of 1 μg/ml Streptavidin-HRPO in PBS/Tween was added and incubated for 30 min at 37°C. 50 μl of developing reagent (0.55 mg/ml ABTS (2,2′-Azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) in citrate buffer ([0.1 M citric acid, pH 4.35]) was added, incubated at 25°C for 15 min, and absorbence at 405 nm was determined. Recombinant IL-2 was obtained from Boehringer Mannheim Corp. (Indianapolis, IN) and was diluted in series to develop a standard curve. Triplicate absorbence values of test samples were thereby converted to lymphokine quantities measured in nanograms per milliliter. Antibodies (capture: JES6-1A12 and detection: biotinylated JES6-5H4) were obtained from PharMingen (San Diego, CA).

Analysis of CD25 and CD69 Expression. 2×10^5 cells were suspended in 50 μl ice-cold PBS/1% calf serum/0.05% sodium azide. Anti-CD25.FITC, anti-CD69, or control Rat IgG FITC antibodies were added, incubated on ice for 30 min followed by two 4-ml washes in PBS/calf serum/NaAzide. 5,000 live gated events were acquired on a FACScan® and the LYSIS II program was used to analyze relevant populations.

Results

CTLA-4 Engagement Inhibits Proliferation and IL-2 Production. We have previously shown that soluble antibodies to CTLA-4 or B7 increased thymidine incorporation and IL-2 production by T cells activated by immobilized anti-CD3 and anti-CD28 in standard 3-d assays (7). These results indicated that blockade of CTLA-4/B7 interactions between the T cells themselves augmented responses by removing inhibitory signals. Since the cultures were assayed at a single time point it was not possible to determine when in the course of the cultures the effect occurred. A kinetic analysis

of the results of CTLA-4/B7 blockade on the proliferation of purified CD4+ T cells is presented in Fig. 1 *A*. As expected from our previous results (7), inclusion of either CTLA-4Ig or Fab fragments of anti-CTLA-4 to cultures stimulated with anti-CD3 and anti-CD28 resulted in an increase in proliferation. The effect was slight at 26 h, at which time there was only marginal proliferation in any of the cultures. At later time points CTLA-4/B7 blockade resulted in a 1.5–2-fold increase in proliferation. The enhancing effect of this blockade was even more apparent at the level of IL-2 production. As shown in Fig. 1 *B*, IL-2 was detectable, although at low levels, in anti-CD3/CD28 stimulated cultures by 26 h. The addition of either anti-CTLA-4 Fab or CTLA-4Ig resulted in an increase of about sixfold in the amount of IL-2 accumulated by 26 h, and nearly ten fold by 40 h.

We also examined the kinetics of the inhibition of proliferation and IL-2 production by cross-linking CTLA-4 together with CD3 and CD28 using antibody coated microspheres. We have previously reported that CTLA-4 cross-linking results in a decrease in thymidine incorporation by T cells stimulated by anti-CD3 and anti-CD28 in 3-d assays (7). This effect is specific to CTLA-4, since irrelevant hamster antibodies (anti-Vγ3) or other antibodies which bind to T cells (anti-L-selectin, anti-Thy1.2) either have no effect or slightly augment proliferation when coimmobilized with anti-CD3 and anti-CD28 (data not shown). The kinetics of thymidine incorporation are shown in Fig. 1 *C*.

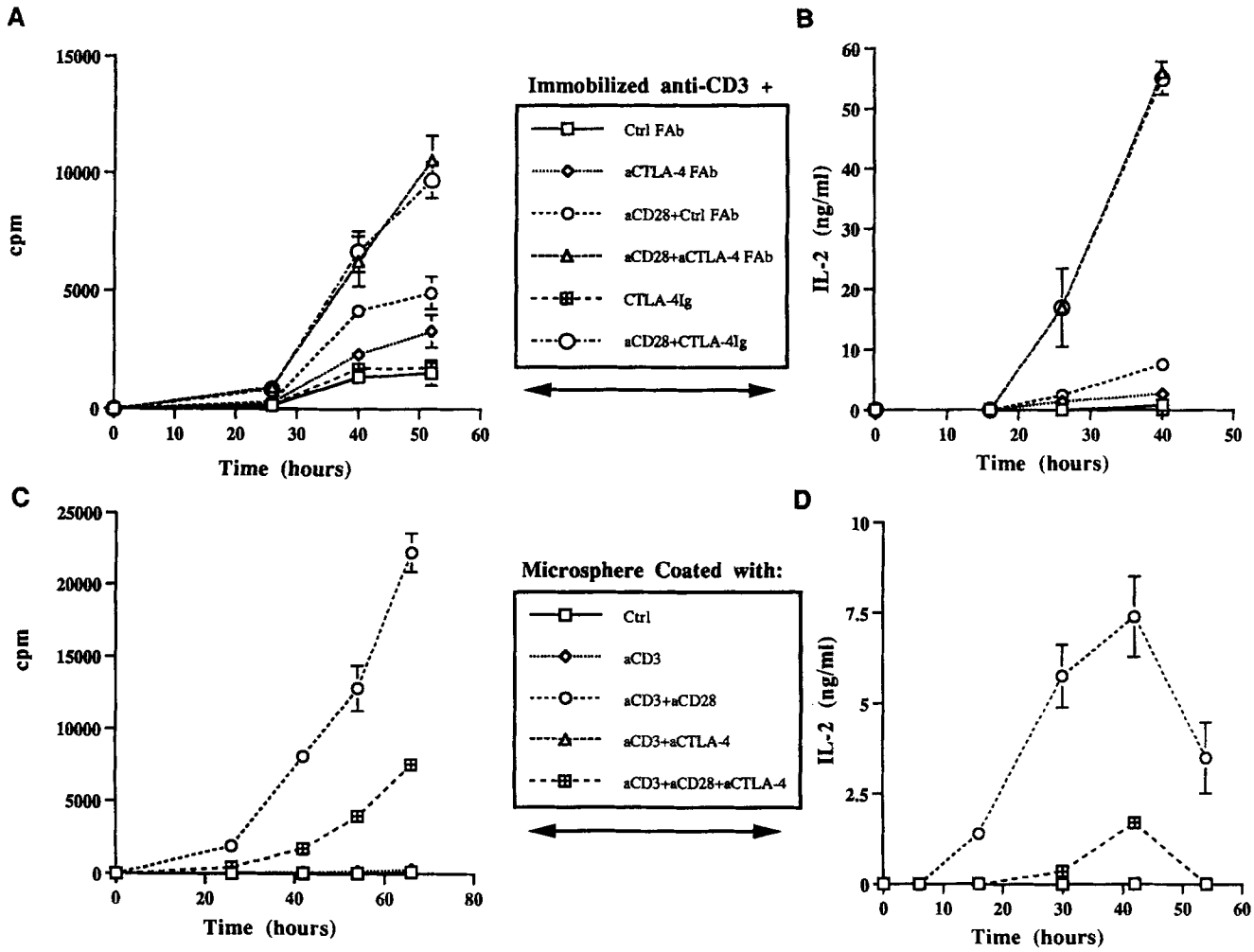

Figure 1. CTLA-4 engagement inhibits T cell proliferation and IL-2 production by anti-CD3 and anti-CD28. (*A* and *B*) Blockade of CTLA-4 engagement inhibits Proliferation and IL-2 Accumulation. CD4+ T cells were cultured in round bottom wells which had been precoated with 50 μl of 0.1 μg/ml solution of anti-CD3. Anti-CD28 was added in soluble form at 10 μg/ml, CTLA-4Ig was added at 5 μg/ml, and control or anti-CTLA-4 FAb fragments were added at 50 μg/ml. (*A*) 12 h before the indicated harvest times, cultures were pulsed with 1 μCi of [^{3}H]thymidine. Triplicate wells were harvested and counted. (*B*) Supernatants were sampled at the indicated times and analyzed for IL-2 using a capture ELISA. (*C* and *D*). Proliferation and IL-2 is inhibited by cross-linking CTLA-4. CD4+ LNT cells were activated with microspheres coated with the indicated antibodies. (*C*) Proliferation was analyzed over the 12 h before the indicated times as described for *A*. (*D*) At the indicated times supernatant was harvested from cultures and analyzed for IL-2 content using an ELISA assay.

Significant incorporation was detectable by 26 h in cultures stimulated by anti-CD3 and anti-CD28. There was essentially no incorporation detectable at 26 h when CTLA-4 was also engaged, and proliferation was three- to fourfold lower in these cultures throughout the assay period. As shown in Fig. 1 *D*, an even more pronounced inhibition of IL-2 production was observed. IL-2 was readily detectable in anti-CD3/CD28 stimulated cultures by 16 h, and increased up to 40 h. When CTLA-4 was also engaged, IL-2 was only barely detectable even after 30 h, and reached a level of only ~1/5 of that in the control cultures at its peak at 42 h.

These results indicate that the inhibitory effects of CTLA-4, whether mediated by its natural ligand or by antibody cross-linking, can be detected early in the course of activation and are not due to precipitous termination of responses at later stages in the process.

CTLA-4 Engagement Does Not Induce Cell Death, But Prevents Cell Cycle Progression. One mechanism that could account for the inhibition of proliferation by CTLA-4 would be the induction or enhancement of cell death. Since the inhibition was detectable throughout the culture period, we assessed the kinetics of cell death occurring in T cell cultures. We initially used hematocytometric counting of cells stained with the vital dye trypan blue to follow the recovery of viable and nonviable cells. The results are shown in Fig. 2. It is significant that the total recovery of cells from the cultures was essentially 100% of input, even in those in which proliferation did not occur. In unstimulated cultures, the number of nonviable cells increases over the culture period, reaching 50% after 54 h. There was a slight increase in the number of dead cells recovered from cultures stimulated with anti-CD3 alone, especially at the earlier time points. Consistent with the proliferation data, cultures costimulated with anti-CD28 yielded an increase in viable cells after 42 h, with a total yield of over 300% at 78 h. Stimulation with anti-CD3 plus anti-CTLA-4 did not result in an increase in dead cells over that observed in unstimulated cultures or in cultures stimulated with anti-CD3 alone. There was also no increase in recovery of dead cells from cultures stimulated with anti-CTLA-4 in the presence of anti-CD3 and anti-CD28 over that of cultures stimulated by anti-CD3 and anti-CD28. It is perhaps significant that throughout the culture period the recovery of viable cells was in fact higher than that from unstimulated cultures or cultures stimulated with anti-CD3 alone. These data indicate that cross-linking of CTLA-4 does not induce cell death, at least as detectable at the level of membrane permeability.

As a more direct and sensitive measure of cell death and cell cycle status, we used propidium iodide staining of permeabilized cells to measure DNA content at various stages in the cultures. Each culture was started with identical numbers of cells, and equal fractions of the cultures were analyzed in order to allow a comparison of the absolute number of recovered cells in the G0/G1, S/G2, and sub-diploid populations. The results are presented in Fig. 3. Total cell recovery was essentially 100% of input or higher under all stimulation conditions. Greater than 99% of input cells were in G0/G1. In unstimulated cultures, the number of cells with sub-diploid amounts of DNA indicative of apoptosis increased to slightly greater than 50% of the total over the course of the culture period. A similar pattern was observed in cultures stimulated with anti-CD3 alone, although slightly higher numbers of cells in S/G2 were obtained. In cultures costimulated with anti-CD28, there was a significant increase in the number of cells in S/G2 as early as 20 h, and this number increased progressively over the assay period. The DNA profiles of cells stimulated with anti-CD3 together with anti-CTLA-4 were essentially the same as unstimulated or anti-CD3 stimulated cultures throughout the assay period with no significant differences in the number of apoptotic cells. However, there were significantly fewer cells in S/G2 in cultures stimulated with anti-CD3 plus anti-CTLA-4 relative to stimulation with anti-CD3 alone. Cultures stimulated with anti-CTLA-4 and anti-CD3 plus anti-CD28 had similar numbers or even fewer cells in the sub-diploid population than any of the other conditions throughout the culture period. Thus there is no evidence of induction of apoptotic cell death by anti-CTLA-4 cross-linking at any time during the course of activation. The main effect of cross-linking CTLA-4 on cells stimulated with

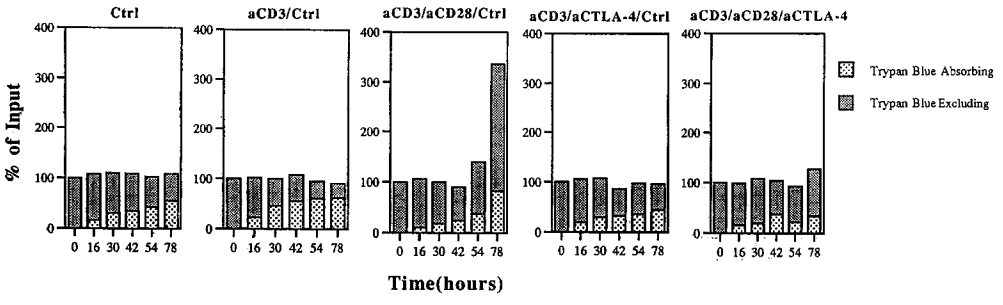

Figure 2. CTLA-4 engagement does not affect T cell viability. Purified T cells (1×10^5 in 200 μl) were activated in round-bottom wells with 1×10^5 of the indicated antibodies immobilized on latex microspheres. After the indicated times, cells were removed, trypan blue was added, and the viable (trypan blue excluding) and dead (trypan blue absorbing) cells were counted using a hemocytometer. 10^{-4} ml of each culture was counted from duplicate wells and the value for this volume was multiplied by 2 to obtain a value for the percent of input (50×10^4 cells/ml was input). Standard deviations of duplicate wells were always less than 10% of the mean and this experiment is representative of four experiments.

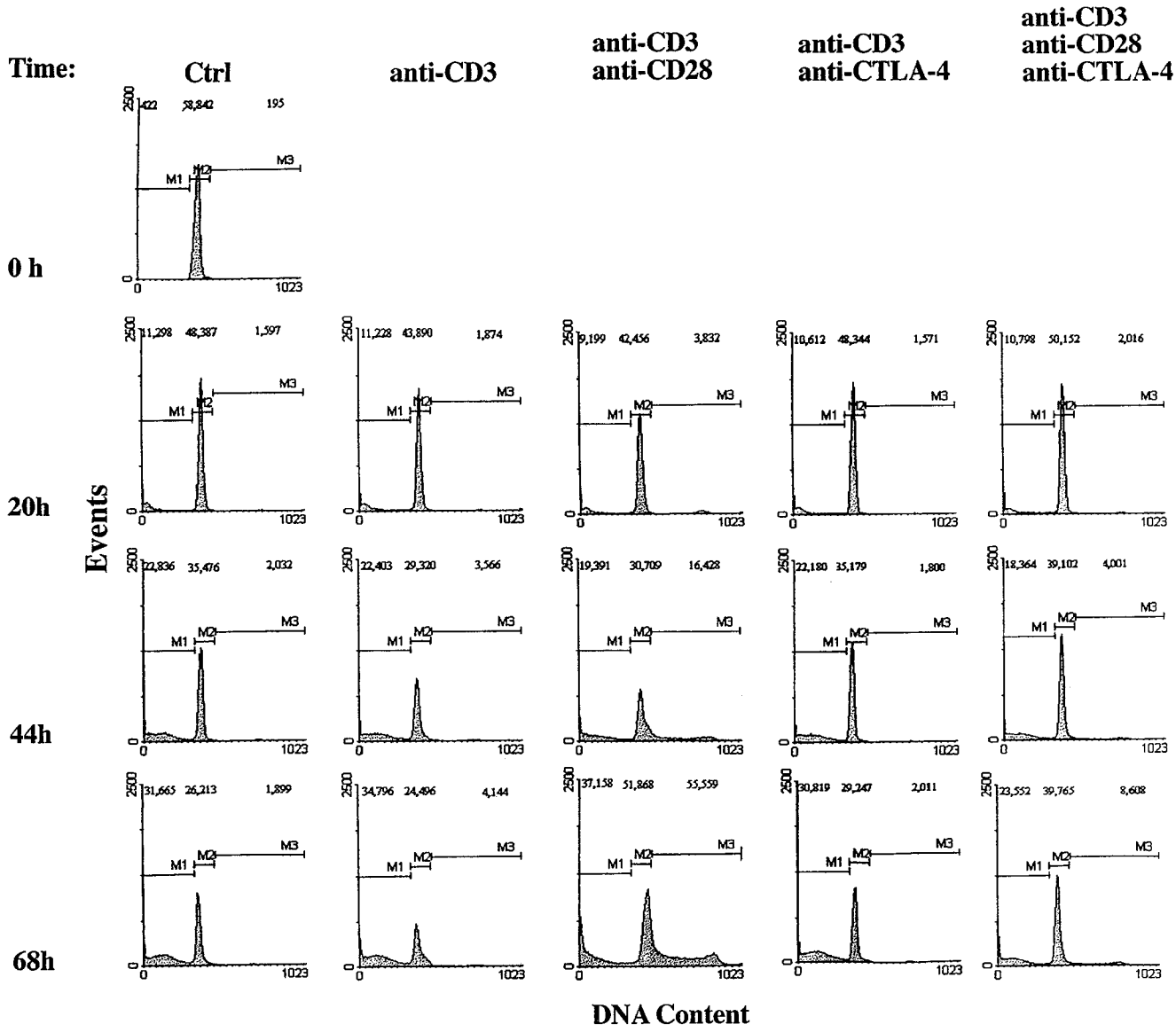

Figure 3. CTLA-4 cross-linking prevents cell cycle entry without inducing apoptosis. Purified T cells (1×10^5 per well in 200 μl) were activated in round-bottom wells with 1×10^5 of the indicated antibodies immobilized on latex microspheres. Cultures were harvested 20, 44, and 68 h after the start of the culture and fixed with 80% ethanol. Samples were all brought up in equal volumes of propidium iodide solution and cells were analyzed for exactly two min at a fixed flow rate. All events above a forward scatter threshold excepting those with forward/side scatter characteristic of microspheres were gated for analysis of PI fluorescence and markers were set for sub diploid (M1), G0/G1 (M2), and S/G2 (M3) quantities to obtain the values for the number of events in each population. This experiment is representative of six experiments.

anti-CD3 and anti-CD28 is an inhibition of the increase in total viable cells, especially those in S/G2. Together, these results indicate that CTLA-4 engagement inhibits cell cycle progression, and an arrest of cells in G0/G1.

CTLA-4 Engagement Partially Inhibits Induction of IL-2 Receptor Alpha Chain Expression. Another hallmark of T cell activation is upregulation of expression of CD25, the IL-2 receptor alpha chain. We used flow cytometry to assess the expression of CD25 on T cells under conditions of CD28 costimulation with and without concomitant CTLA-4 ligation. As shown in Fig. 4, stimulation of T cells with anti-CD3 alone resulted in the induction of expression of CD25 on ~60% of T cells within 24 h. Costimulation with anti-CD28 increased this expression with respect to both the number of positive cells and the level of expression at 24 h, and the expression was further enhanced at 60 h of culture. When CTLA-4 was also engaged, CD25 expression was expressed by a smaller fraction of the cells (47% vs. 80%) and the mean level of expression was much lower at 24 h (MFI 162 vs. 194) and at 60 h (MFI 332 vs. 669) relative to cultures costimulated with anti-CD28. This data demonstrate that CTLA-4 engagement inhibits the upregulation of CD25 throughout activation. The mechanism for the inhibition is not certain. Since IL-2 has been shown to up-

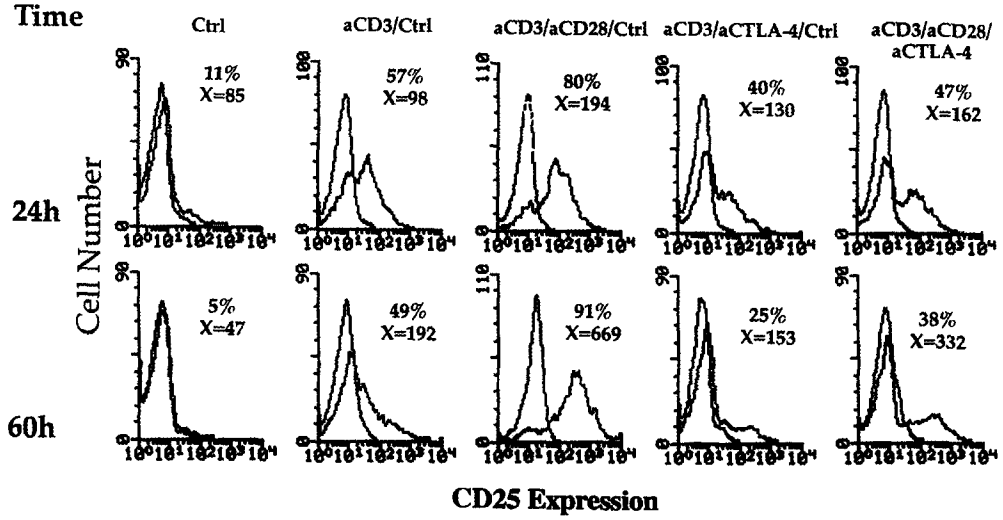

Figure 4. Expression of CD25 on activated T cell populations. Purified CD4+ T cells were activated using microsphere-immobilized antibodies as described in the legend to Fig. 3. After the indicated time, cells were removed, washed once with PBS/1% calf serum and stained with either anti-CD25.FITC or control Rat IgG.FITC. Cell populations were analyzed by flow cytometry. Markers were set for the negatives and the mean fluorescence of cells above that threshold (X=), and percentage of cells staining above that threshold was calculated using the Lysis II program.

regulate CD25 in human peripheral T cells and in murine T cell clones (25, 26), inhibition of its expression may be an indirect consequence of the inhibition of IL-2 production by CTLA-4. On the other hand, it has been recently reported that CD28 costimulation can directly induce CD25 expression on virgin murine T cells by an IL-2 independent mechanism (27). Our previous observation that IL-2 did not fully restore proliferation to cultures inhibited by CTLA-4 (7) implies that CTLA-4 may directly interfere with this process. Whatever the mechanism, our results demonstrate that CTLA-4 engagement can interfere with another early feature of T cell activation, CD25 upregulation.

CTLA-4 Engagement Partially Inhibits Expression of the Early Activation Marker CD69. CD69 is an early and transient marker of T cell activation (28). In Fig. 5 we present a kinetic analysis of the effects of CD28 and CTLA-4 engagement on induction of CD69 expression. At 12 h, CD69 was expressed by greater than 50% of T cells activated with CD3 alone or costimulated with anti-CD28, while fewer than 15% of costimulated cells also subjected to CTLA-4 ligation were positive. At 24 h, CD69 expression was detectable, albeit in a heterogeneous pattern, on greater than 75% of CD28 costimulated cells. At this point fewer than 45% of cells from cultures in which CTLA-4 had also been engaged expressed CD69 and the level of expression was reduced. By 36 h, CD69 expression had returned to essentially resting levels in all the cultures. Thus, it appears that CD28 costimulation augments and prolongs CD69 expression, whereas CTLA-4 ligation inhibits the initial upregulation of CD69. This result is consistent with the observation that CD69 levels were found to be constitutively elevated on T cells isolated from CTLA-4-deficient mice (10, 11) and provides additional evidence suggesting a role for CTLA-4 in preventing the early induction of T cell activation.

Discussion

While it has become apparent CTLA-4 has an important role in downregulating T cell responses, the mechanism by which this is accomplished is not at all clear. It has been suggested that CTLA-4 terminates responses of activated T cells, perhaps by induction of apoptotic cell death (14, 15). Here we have demonstrated that CTLA-4 mediates inhibition of proliferation and IL-2 production by resting T cells in the absence of CTLA-4-mediated cell death. We found that the recovery of viable and nonviable cells from anti-

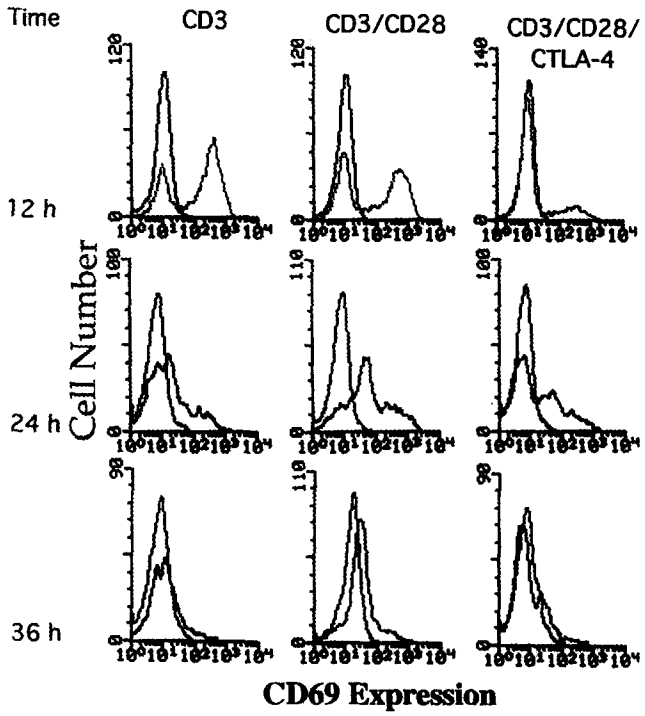

Figure 5. Expression of CD69 on activated T cell populations. Purified CD4+ T cells were activated using microsphere-immobilized antibodies as described in the legend to Fig. 3. After the indicated time, cells were removed, washed once with PBS/1% calf serum, and then stained with either control RatIgG FITC or with anti-CD69 FITC. 5,000 live gated events were collected for each sample. This experiment is representative of three individual trials.

CTLA-4-inhibited cultures is similar to that observed in control antibody or anti-CD3 stimulated cultures. We found no evidence for the accumulation of cells with sub-diploid quantities of DNA associated with apoptotic cell death even 1–2 d after inhibitory effects of CTLA-4 cross-linking are first observed at the level of proliferation and IL-2 production. Finally, CTLA-4 cross-linking arrests T cells in a G0/G1 phase of the cell cycle. Taken together, these data clearly demonstrate that inhibition of T cell proliferation and IL-2 secretion by CTLA-4 can occur in the absence of cell death.

At least two possible explanations may be offered to account for the discrepancy between our results and an earlier report of apoptotic cell death resulting from CTLA-4 ligation (15). This study examined the effect of CTLA-4 ligation upon restimulation of a long-term human T cell clone whereas we have examined the effect of CTLA-4 on freshly isolated murine T cells. Another possibility is that CTLA-4 might have indirectly contributed to the death of the T cell clone by depriving it of an essential growth and survival factor by the cessation of IL-2 production. This possibility is supported by the observation that the apoptotic death in these clones was prevented by the addition of exogenous IL-2 (15).

An important implication of the data presented here is that CTLA-4 may have a role in regulating T cell responses at early stages in the process. CTLA-4 has not been demonstrable by flow cytometry on resting T cells, and reaches maximal levels only 1–2 d after activation (5–7). This has led to the notion that CTLA-4 is an activation antigen whose main role is to terminate ongoing responses (3, 12). Our data do not reveal a precipitous termination of ongoing responses, but rather an inhibition and delay of events associated with the progression of T cell activation. We observed that inhibition of the upregulation of the activation marker CD69 was detectable by 12 h, inhibition of IL-2 production was observed by 16 h, and the initiation of proliferation at 24 h was delayed. Despite the fact that CTLA-4 expression has been undetectable by the relatively insensitive method of flow cytometry until later after activation, our assays reveal the presence of functionally significant levels much earlier than has been appreciated.

These observations lead us to speculate that CTLA-4 may have an important role in regulating both initiation and termination of T cell responses. Even though it is initially expressed at very low levels, the higher affinity of CTLA-4 for the common B7 ligands may give it a competitive advantage over CD28 for binding when these ligands are limiting. Thus, when a T cell encounters an antigen on presenting cell which express only modest amounts of B7, for example a resting B cell (29), CTLA-4 may dominate and prevent the initiation of the response. This notion is consistent with our observation that the level of B7-2 expressed by T cells themselves is insufficient to provide costimulation for anti-CD3 stimulated cells, but can at least partially inhibit CD28-mediated costimulation ((reference 7) and Fig. 1 *A*). However, in an encounter with an antigen presenting cell with high levels of B7 expression, such as an activated B cell, macrophage, or dendritic cell (29, 30), the level of CTLA-4 would be limiting allowing the costimulatory activity of CD28 to dominate and the activation to proceed. With time and the upregulation of CTLA-4, the inhibitory effect would again become dominant and the responses terminate. CTLA-4 mediated termination of T cell responses may allow some cells to evade activation induced cell death (31) and become memory cells, ready to again respond to antigen upon the decay of CTLA-4 expression.

The precise role of CTLA-4 in regulating T cell functions in the early and late stages of responses remain to be established. However, our data clearly demonstrate that CTLA-4 engagement can counteract the costimulatory effect of anti-CD28 on multiple aspects of the activation of resting T cells. CTLA-4 mediated inhibition of T cell proliferation is a consequence of cell cycle arrest, perhaps as a consequence of inhibition of IL-2 production, and is not due to induction of cell death.

We are grateful to Stan Grell for assistance with tissue culture and Peter Schow for assistance with cell cycle analysis. We thank Drs. Cynthia Chambers, Michael Kuhns, and Delanie Cassell for helpful discussions and critical reading of the manuscript.

This work was supported by National Institutes of Health grants CA40041 and CA09179.

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Received for publication 1 March 1996 and in revised form 1 April 1996.

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